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Estimation of starch in plant tissue

Robert Delafield Powell
Iowa State College

NOTE TO USERS

ESTIMATION OF STARCH IN PLANT TISSUE

by

Robert Delafield Powell

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INTRODUCTION

Determination of starch has been of interest to biological chemists, plant physiologists and food technologists for over a century. Reports on the determination of starch are found in the literature as far back as 1831. Plants are composed of a great many different types of polysaccharides which might interfere with the determination of starch. The number and type of these compounds vary tremendously from one species of plant to the next. The food technologist is usually working on a particular species, and, therefore, may use a method that is applicable to that type of plant without consideration of its adaptability to other plants. The biochemist and plant physiologist, however, are interested in a large number of different species. It is necessary for these research workers to have available a method that may be applied to plants in general. The work presented in this paper was done with this objective in mind.

Probably the most prevalent method of starch determination used by plant physiologists is that of Loomis and Shull (17). This method, starting with dry material from which sugars have been removed, consists of an extraction with 10 percent alcohol to remove dextrins. Water is added to the residue and heated to gelatinize the starch, which is then digested

with saliva and extracted with water. Neutral lead acetate is added to remove gums, and the solution filtered and deleaded. An aliquot of the clear solution is hydrolyzed with hydrochloric acid in a concentration equal to 1.0 ml concentrated acid for each 20 ml of solution (1 + 20). Dextrins may be determined by application of the same procedure to the 10 percent alcohol extract, eliminating the gelatinization and enzymatic hydrolysis. The resulting glucose is determined and the starch calculated by using a conversion factor of 0.90. The work presented here will be a study of the various phases of this method.

REVIEW OF LITERATURE

One of the oldest methods for the estimation of starch is that of Hermbstaedt (13) which appeared in 1831. This method was essentially the mechanical isolation and gravimetric determination of starch. Present day methods consist of some means of freeing starch or its hydrolytic products from the remainder of the plant tissue and the determination of the starch or sugar content of the extract. It has also been stated that preliminary treatment of the plant material is of great importance.

Grinding of Plant Material

The fineness of grinding necessary to extract starch quantitatively from plant tissue has been the subject of considerable disagreement among various authors, probably due to various methods of extraction and differences in plant tissue. Sullivan (26), working with apple twig terminals and a calcium chloride extraction, found that the material must be able to pass through an 80 mesh screen, and recommended that it pass through a 100 mesh screen. Hassid, McCready and Rosenfels (12), using hot, dilute, alcoholic hydrochloric acid as a preliminary extractant, claimed that grinding to pass through a 50 to 60 mesh screen was sufficient.

Griffiths and Potter (10) state that difficulty in extraction of starch may be partly due to inadequate grinding. They did not, however, give any experimental data.

No data have appeared in the literature concerning the effect of prolonged grinding on starch. Schoch (24), however, stated that prolonged dry grinding of starch in a pebble mill eventually yielded a product that stained red with iodine. He believed that hydrolytic and oxidative changes may occur, as well as dextrinization due to heat generated in the grinding process. He further stated that mechanical rupture of the bonds in a molecule such as starch is entirely possible, and that a similar phenomenon was found with proteins.

Extraction of Starch

Starch is found in plants in a great many different types of tissues. It is always formed inside a plastid in a living cell. These are usually parenchyma cells and therefore thin walled. However, these cells may be surrounded by woody tissue, as in the xylem of the root and stem. The starch must be removed from the cells and freed from any woody material surrounding these cells. Several means of extraction have been proposed.

Probably one of the oldest means of extracting starch takes advantage of the hydrolytic activity of enzymes.

This, in many cases, is coupled with a subsequent acid hydrolysis. With these techniques, it is necessary to determine the hydroltyic products formed and calculate the starch. Davis and Daish (5) showed that, under proper conditions, digestion of starch with taka-diastase yields glucose and maltose only. They then determined the glucose and maltose polarimetrically and by copper reduction. The quantity of each was calculated by simultaneous equations and the amount of starch estimated. Several papers then appeared with conflicting results on the use of taka-diastase for the determination of starch. Horton (14) suggested that the hydrolysis to glucose and maltose is not complete and that some dextrin remains. Collins (4) reported glucose values of 98.5 to 101.1 percent by hydrolysis of starch when 50 ml of 0.3 percent starch solution was incubated at 40°C for 36 hours with 1 ml of 10 percent taka-diastase and 5 ml of acetate buffer (pH 5.0). Widdowson (28) could not find a constant ratio of glucose to maltose with enzymatic hydrolysis. These discrepancies were explained by Denny (6). He used dialyzed taka-diastase and showed that the proportion of glucose to maltose to dextrin depended upon the concentration of enzyme used and the conditions of the incubation. In the same paper he also showed that the postulation of the destruction of glucose by acid hydrolysis was invalid. He showed that the previous data did not merit

such a conclusion, and was himself not able to find any destruction of glucose until he used a concentration of 27 ml of hydrochloric acid (sp. gr. 1.19) in a total volume of 110 ml of acidified glucose solution (volume after adding acid), heated in a boiling water bath for 2.5 hours. He still expressed concern that acid hydrolysis of the enzyme extract might lead to high results from the hydrolysis of water soluble, non-starch substances. Denny did not clear his extracts before acid hydrolysis. He did test the effect of preheating on the effectiveness of taka-diastase, and found that at least momentary heating at 60°C was necessary for complete enzymatic digestion. Examination of photo-micrographs taken with polarized light showed that the treatment resulted in changes in structure of the starch grain, rather than in gelatinization. This work, however, was evidently done on pure starch, and much more rigorous treatment might be expected to be necessary if the material were in a refractory plant tissue.

Hassid, McCready and Rosenfels (12) used salivary diastase for the hydrolysis of starch. With this enzyme they found that a "hydrolysis limit" of 0.890 mg of maltose per mg of anhydrous starch could be obtained in two hours. The saliva was diluted one-half and filtered. It was stated that this preparation could be kept for at least two weeks with refrigeration. The limit value reached was found

to be constant with a variety of plant material. They were not able to obtain any hydrolysis of cherry gum, a glucosan, two bacterial polysaccharides or alpha- and beta-methyl glucoside with saliva.

The use of mineral acids has been advocated for the extraction of starch without hydrolysis. Rask (23) found that 20 gm of concentrated hydrochloric acid per 100 ml of solution would extract starch quantitatively from plant material. Denny (7) studied the effect of hydrochloric acid at different times of contact and at different concentrations under the same conditions. He found, using potato starch, that he could obtain nearly 100 percent recovery with both Rask's hydrochloric acid (23) and with sulfuric acid at a concentration of 32.5 to 37.5 percent, but only if the temperature did not rise above 22.5°C and the contact was not longer than one hour. With higher temperatures and longer time periods there was a definite loss of starch. He believed that the Rask method was not valid as a general procedure, since it was found to give high values for such tissue as orange rind which gave a negative test for starch. In addition, he stated that there was some danger of incomplete extraction of the starch.

The use of salts to extract starch has also had a rather wide application. The first method of this sort that received any widespread attention is that of Denny (6)

which was based on a method originally proposed by von Fellenberg (8). In this procedure, the starch was extracted by hot calcium chloride and precipitated as the starch-iodine complex. In a comparison with other methods, Denny claimed that calcium chloride gave more accurate results, since he was able to obtain negative results with tissue that did not show a qualitative test for starch. The other methods gave positive values under these conditions. In a second paper (7) he found that this procedure, using calcium chloride, gave comparable results with hydrochloric acid (Rask's acid) or sulfuric acid extractions, but he still seemed to prefer calcium chloride. Sullivan (26) claimed that two possible errors were involved in using this reagent. It was difficult to extract plant tissue completely with the calcium chloride and to obtain a quantitative precipitation by iodine after the extraction was achieved. Complete extraction was possible by boiling for 50 to 60 minutes with 40 to 50 ml of saturated calcium chloride with a total volume of 60 ml maintained throughout the boiling period. The starch-iodine precipitate could not be held on the filter (asbestos pad) when the complex was formed in the presence of calcium chloride. He therefore introduced a double precipitation of the extracted starch with alcohol, washing the precipitate free of salt, suspension of the starch in water, and finally, re-precipitation with iodine in the

presence of ammonium sulfate. The starch-iodine complex was easily retained when formed in the presence of ammonium sulfate. The double precipitation gave somewhat lower results than were obtained with taka-diastase, a factor which Sullivan attributed to digestion of non-starch materials by the enzyme. Pucher and Vickery (22) claim that starch is not stable in saturated calcium chloride for more than 48 hours, and is even less stable when the solution is diluted with ten parts of water. Further investigation showed that a calcium chloride suspension of starch was very sensitive to acid and that the calcium chloride solution became acid upon heating with plant tissue. Marked losses of starch were found to occur if the pH fell below 6.0. The amount of loss varied with the tissue used. The addition of magnesium carbonate preserved a neutral solution and prevented the loss of starch. Pucher and Vickery recommended a temperature of 100°C instead of boiling saturated calcium chloride (113°C). However, with all factors considered, they preferred cold concentrated hydrochloric acid as an extractant.

Nielsen (18) introduced the use of perchloric acid as an extractant for starch. He found that a concentration of nearly four molal was necessary to bring the starch into solution, and that no hydrolysis occurred with potato starch in contact with acid at a concentration of 4.8 molal for

15 minutes. The addition of the perchloric acid caused some heating which aided in the solubilization of the starch. Pucher, Leavenworth and Vickery (21) used perchloric acid following a gelatinization brought about by heating a suspension of plant tissue in a boiling water bath for 15 minutes. No data are given regarding increased time or the temperature of gelatinization. They recommended two successive extractions with perchloric acid. Concentrated (72 percent) perchloric acid is an extremely dangerous reagent in the presence of organic matter. The procedure calls for addition of the acid at this strength to a suspension of the plant material. Whereas perchloric acid is a useful reagent, it is dangerous and should be used only by an experienced analyst.

Determination of Starch

After starch (or its hydrolytic products) has been extracted from plants, it is necessary to determine in some way the quantity of starch represented by this extract. This may be done in one of four ways: (a) direct weighing of the extracted starch, (b) determination of the hydrolytic products of starch, (c) measurement of the specific rotation of the starch molecule or (d) colorimetric determination of the starch-iodine complex.

Rask (23) used the direct weighing method for determining starch. After extraction with cold, concentrated hydrochloric acid, the starch was precipitated with alcohol, dried and weighed. This method was designed for cereal products, which are high in starch and low in impurities, and it obviously would not be applicable to material low in starch unless large samples were used. Denny (7) found that in most cases non-starch substances were included in the alcohol precipitate.

The hydrolysis of starch may be carried out in three different ways: (a) heating with acid, (b) treating with enzymes or (c) enzymatic digestion followed by heating with acid. All of these methods have been employed to obtain the hydrolytic products of starch.

Wherever starch is found in plant tissue there are various quantities of other acid hydrolyzable carbohydrates. For this reason, methods of determining starch through direct acid hydrolysis of the material, without a previous isolation or enzymatic hydrolysis of the starch, have been generally abandoned. The most recent attempt at such a method was made by Fraps (9). He heated plant material with 0.02 N hydrochloric acid in a water bath for 2.5 hours, and found with the materials used, that pentosans amounting to 1.78 to 6.64 percent were determined as starch.

Many methods have used acid hydrolysis to convert starch

to glucose after the starch has been extracted and partially purified. Pucher, Leavenworth and Vickery (21) used such an acid hydrolysis following a perchloric acid extraction and precipitation with iodine. They claimed that this was more accurate and general than the determination of the starch through its physical properties, for reasons that will be discussed later.

Enzymatic hydrolysis directly on the plant tissue has been used in several methods (4, 5, 6 and 28). These methods involve long incubation periods, usually with taka-diastase. Denny (6) used a 44 hour incubation under controlled conditions and thought that the results obtained were high. Other workers (5, 28) have not attempted to obtain complete hydrolysis but have calculated the starch from the maltose and glucose formed. Denny (6) used taka-diastase instead of acid to hydrolyze starch after it had been extracted by calcium chloride. He gave no data comparing acid and enzyme hydrolysis following a calcium chloride extraction. Hassid, McCready and Rosenfels (12) used saliva (pH of 5.6) for two hours at 37-40°C and determined the maltose formed after the solution was cleared and deleaded. They claimed that no error was introduced by deleading before filtering.

Acid hydrolysis following digestion with enzymes, involves essentially the extraction of starch with the enzyme, which has been discussed. The method has no advantage

over the use of the enzyme alone except that a shorter incubation period may be used and a single product, glucose, may be determined. Otherwise, the method is subject to the same criticisms as the enzymatic digestion without the use of acid. Sullivan (26) compared the use of taka-diastase plus acid hydrolysis with the procedure described by Denny (6) using taka-diastase directly on the plant tissue, and found that the use of acid gave higher results.

The figure used to convert the quantity of glucose formed to starch has been the subject of some controversy. Sullivan (25) calculated the value on a theoretical basis. The figure approaches 0.90 as the number of glucose units increases. With 24 glucose units, the theoretical figure would be 0.904. If the molecule were cyclic, the figure would be exactly 0.9. Noyes et al. (20) claimed that incomplete hydrolysis was obtained, and that under prolonged heating some destruction of glucose occurred. For these reasons they suggested a conversion factor of 0.93. Even though this figure is empirical in nature, several workers have accepted it. In the interpretation of results of a starch determination, it is, therefore, necessary to consider the conversion factor that was used.

Pucher and Vickery (22) developed a method for starch determination in which the quantity of starch was obtained

colorimetrically. In this procedure the starch was extracted and precipitated as the starch-iodine complex. The complex was decomposed and the starch dissolved in dilute acetic acid. The starch-iodine complex was re-formed, and the intensity of the color was determined spectrophotometrically. A calibration curve was made using potato starch and the milligrams of starch read directly from the curve. In a later paper, Pucher, Leavenworth and Vickery (22) abandoned this technique in favor of the determination of glucose following acid hydrolysis. The change resulted from a better understanding of the chemical nature of starch.

Bates, French and Rundle (2) were able to show the presence of two distinct fractions of starch. These fractions were previously named amylose and amylopectin[1]. Iodine stains the amylose fraction blue and the amylopectin fraction purple to red. They showed further that the amylose fraction was able to bind much more iodine than the amylopectin. Using this difference between the two fractions they were able to determine the percentage of amylose in starch from several different sources. They found that the amylose content varied from zero percent for waxy corn to 34 percent for

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[1]Names and definitions according to structure are those of Meyer (original reference unavailable) and adopted by Bates, French and Rundle (2).

lily bulb. Baldwin, Bear and Rundle (1) found that the wave length for the absorption maximum of the amylose-iodine complex differs with amylose from different plant sources. The differences were characteristic of the source. In addition, there were rather large differences for the molecular extinction coefficient of the different amylose-iodine complexes. In fact, the extinction coefficient was used as a rough test of the molecular size.

Nielsen and Gleason (19) attempted to reconcile the use of potato starch for the determination of a standard curve to be used in the colorimetric estimation of other starches. A series of empirical conversion factors were determined for starches from several different plant tissues. Such factors are probably satisfactory for routine work, but would not be applicable in a general method. As Pucher, Leavenworth and Vickery (21) point out, a factor would have to be determined for each tissue tested, and even then there is no assurance that this factor would be constant at different stages of maturity or under different growing conditions, since the factor would be a function of the size of the amylose molecule and the amylose-amylopectin ratio.

A great many methods of determining starch through the use of polarimetry have appeared in the literature. Clendenning (3) has recently proposed such a method which consists of an extraction of the starch, precipitation of

the proteins and an estimation of the starch content by polarization. This method was designed for cereal products and may be satisfactory for this purpose, but since the optical rotatory power is dependent upon the amylose-amylopectin content, the method is open to the same objections as the colorimetric method.

MATERIALS AND METHODS

This thesis deals with a method for estimating starch, which will be developed in later sections of the paper. Only the materials and the general methods used will be given in this section.

Plant Materials

The plants used in this study were chosen to represent varying tissue types and because qualitative tests showed them to contain at least a moderate amount of starch. The following plants were used: (a) *Abutilon theophrasti* leaves, stems (bases and tips) and roots, (b) *Portulaca oleracea* stems, (c) alfalfa (*Medicago sativa*) roots, (d) apple (*Pyrus malus*) stems and (e) tomato (*Lycopersicum esculentum*) stems. Collection of these plants was made in the late summer and fall of 1948. In addition, onion (*Allium cepa*) bulbs and turnip (*Brassica rapa*) roots were purchases from the grocery store in the summer of 1950. The latter two were chosen because they were known to contain no starch. All of the tissues were brought immediately to the laboratory, cut into small pieces, put into hot 95 percent alcohol and brought to a boil. The alcohol containing the tissue was allowed to boil for a few minutes and cooled. After filtering off the

alcohol, the tissue was dried at 100°C and ground in a Wiley mill using a 20 mesh screen. The ground material was extracted by the soxhlet method for 24 hours with 80 percent alcohol to remove low molecular weight carbohydrates. After air drying, the material was placed in an oven at 100°C for one to two hours. The Abutilon leaves and apple stems used in many of the experiments described were further ground in one of two ways: (a) a ball mill for 12 hours using 20 gm and 20 small rounded pebbles in each grinding, and (b) a micro Wiley mill with a 40 mesh screen for the Abutilon leaves, and an 80 mesh screen for the apple stems.

The pure starch used in the experiments described in this work was isolated from potato tubers. The tubers were washed and rubbed through a plastic food grater into water. The liquid was decanted off and the residue washed by decantation until only starch grains could be observed microscopically. The starch was then washed with 80 percent alcohol and finally with ether. It was filtered through a Buechner funnel and sucked dry. The purity of the preparation was found by determining the glucose formed by direct acid hydrolysis of the starch. A factor of 0.90 was used to convert to starch. By this technique, the preparation assayed 89.5 percent starch. A blank determination, in which the acid hydrolysis was omitted, showed no reducing

substances. Kjeldahl analyses[1] showed no nitrogen present.

Enzymes

In most of the experiments described, saliva was used as the source of diastase. Secretion of saliva was stimulated by chewing paraffin. The saliva was collected and filtered through a Whatman number 30 filter paper. Although Hassid, McCready and Rosenfels (12) found that such a preparation would keep for two weeks if refrigerated under toluene, fresh saliva was used each day. The taka-diastase was a special, glucose free, investigational preparation obtained from Parke Davis and Company. It was stated as having 650 percent activity and was numbered 487546.

Equipment

The work described in this paper required no equipment other than that found in most plant physiology laboratories. In the grinding experiments discussed later, various milling processes were used. The types of mills used are described as follows:

Wiley Mill No. 1, with a coarse screen

Micro Wiley Mill, with various screens

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[1] Analyses performed by M. M. Abul-Ela

Ball Mill, consisting of porcelain jars in which rounded flint pebbles of about one inch in diameter were used for grinding

C and M Laboratory Mill No. 8; This is a hammer type mill which has four rapidly rotating arms that shatter the material, which drops through a screen into a bag.

In the glucose determination, it is necessary to heat the sample in a boiling water bath. A rack holding 24, 25 mm tubes and a water bath of convenient design were used for this purpose.

Analytical Methods

The method of glucose determination used throughout this work was that described by Hassid (11). This method was chosen because of its simplicity and because it has recently been studied by Lind (15 and 16) in this laboratory. The procedure consists of reduction of ferricyanide by heating with a glucose solution in a boiling water bath. The mixture is cooled, acidified and the reduced iron titrated with ceric sulfate using setopaline-C as an indicator. With each batch of tubes, a standardization and blank were run. A glucose solution of known strength was used for the standardization, and an equal volume of distilled water served as the blank. The milliliters of ceric sulfate per milligram of glucose was calculated, and this value was used in calculating the glucose derived from the

starch in the samples. The starch was calculated by multiplying the glucose value by 0.90. All data reported are averages of closely agreeing duplicates.

EXPERIMENTAL RESULTS

In some instances the experiments presented here may not appear to be in logical sequence. Such a logical sequence, however, is difficult to determine since what is done in one phase of the method may effect other phases. For the most part, the order of presentation is the time sequence in which the experiments were done, and, unless otherwise stated, the results of such an experiment are incorporated in subsequent work. In some cases it will be noted that the results do not check among various experiments. These inconsistancies are due to variations of conditions between different experiments. Such variations are smaller within a single experiment and therefore the results of each experiment are comparable.

Enzyme Studies

Several experiments were performed to determine the effect of concentration of enzyme and time of incubation on the extraction of starch with saliva and taka-diastase.

Effect of concentration of saliva

Approximately 0.4 mg samples of Abutilon leaves, ground in a ball mill for 12 hours or ground in a Wiley mill with

a 40 mesh screen, were weighed into 100 ml volumetric flasks and suspended in 40 ml of distilled water. The material that was ground in the Wiley mill was difficult to wet, and these samples were suspended by a vacuum infiltration technique in which 20 ml of water was added and the material subjected to a vacuum while the flask was being shaken. The vacuum was released and the process repeated until all the material was in apparent suspension. A second 20 ml of water was added, rinsing down the sides of the flask. The samples were autoclaved for 60 minutes at 15 pounds pressure to gelatinize the starch. After cooling the suspensions in a water bath, 3 ml of saliva was added to each sample. Fresh, filtered saliva was used and diluted to the strength desired for the particular sample. Between 1 and 2 ml of toluene were added, the samples shaken vigorously, and the sides of the flask rinsed with 10 ml of water. All of the samples were then allowed to incubate for two hours at 30°C. At the end of this time, 2 ml of neutral lead acetate was added, and the samples were diluted to the 100 ml mark with water. The samples were filtered through Whatman, number 30, filter paper into a flask containing a small quantity of solid $K_2HPO_4.3H_2O$. Approximately 1 ml of the sample was tested for complete precipitation of lead by adding a drop of the lead acetate reagent. If this test did not show an excess of phosphate (indicating removal of lead), more

solid $K_2HPO_4 \cdot 3H_2O$ was added. A 40 ml aliquot of the clear solution, obtained by centrifuging, was pipetted into a 50 ml volumetric flask, and 2 ml of concentrated HCl added. The flasks were shaken and autoclaved for 60 minutes at 15 pounds pressure. After autoclaving, the samples were cooled, neutralized with sodium hydroxide and diluted to volume. Sugars were determined on a 5 ml aliquot, and the percentage starch was calculated. For each dilution of saliva, a blank determination was made using boiled saliva of the same concentration and eliminating the HCl hydrolysis. The blanks were heated in the autoclave and cleared in the same way as the samples. The experiment was repeated as described using the same and other dilutions of saliva. In this case, 1 ml instead of 2 ml of the lead acetate solution was added. The results shown in table 1 and figure 1 are corrected for the blank. The blank was found to be consistant among the different dilutions, which shows that no reducing substance was introduced by the saliva. Therefore, the average of the blanks was used in making the correction.

The data in table 1 and figure 1 show that with a two hour incubation, only a small quantity of saliva is necessary. The rather poor agreement between experiments in this case, is probably due to two factors. First, the error is magnified because the spacing of the units on the ordinate is

Table 1. The effect of the concentration of saliva used in the determination of starch in Abutilon leaves.

Ml saliva per 106 ml of suspension	Percentage starch		
	Ground in ball mill	Ground in Wiley mill	Avg.
0.0	2.6	1.4	2.0
0.024	8.4	7.2	7.6
0.024	7.1		
1.20	8.5	7.4	7.7
1.20	7.3		
3.0	8.0	7.3	7.6
6.0	8.9	8.0	8.1
6.0	7.6	7.8	
6.0	8.0	—	—
Avg. treated	8.0	7.5	7.8

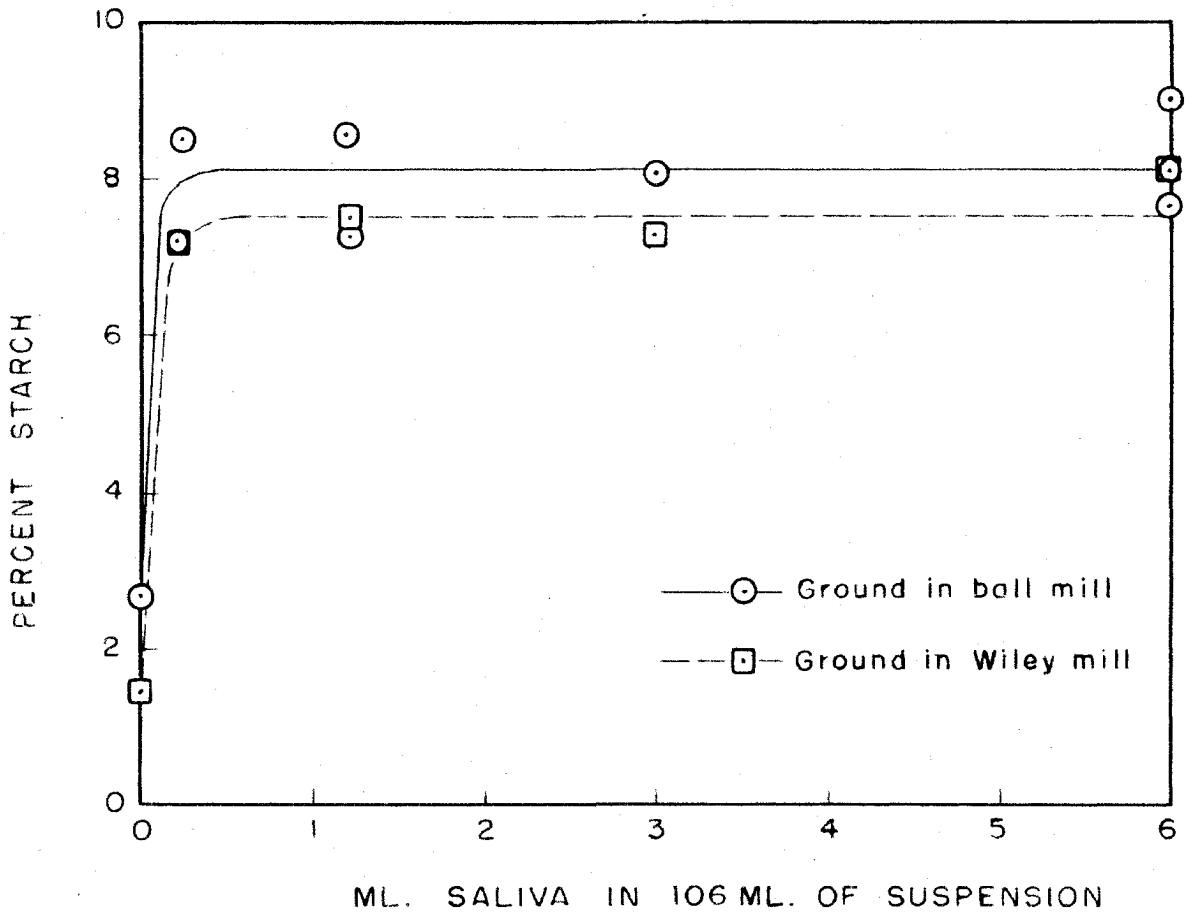

Fig. 1. The effect of saliva concentration on the determination of starch in Abutilon leaves.

larger than the smallest experimental error that could be expected, and second, because no precautions were taken to limit the excess of phosphate, a factor which was later found to be important.

Effect of time of incubation with saliva

Two types of experiments were performed to determine the optimum time of incubation with saliva. Apple stems, ground in a micro Wiley mill with an 80 mesh screen, and Abutilon leaves, ground in the same mill using a 40 mesh screen were used. The first experiment was done in the same manner as that described above, except that a constant dilution of 1 to 50 of saliva was used, and the time of incubation was varied from zero to 20 hours. With the zero time samples, 1 ml of lead acetate was added before addition of the enzyme. This experiment was repeated in a different manner using various enzyme concentrations. Approximately 3 gm of the plant material was weighed into a 500 ml volumetric flask, suspended in 40 ml of water, gelatinized and cooled as previously described. To these samples, saliva to make the desired concentration, was added, and the flasks made up to volume. After addition of toluene, the flasks were shaken vigorously and the contents emptied into 500 ml Ehrlenmeyer flasks and incubated at 30°C. At the end of the desired time interval, the flask was shaken,

and duplicate 40 ml aliquots were pipetted into 100 ml volumetric flasks containing 1 ml of saturated neutral lead acetate solution. Care was taken to remove a proportionate share of the solid material each time. These samples were filtered, deleaded and carried through the determination as described before. The results are shown in tables 2 and 3 and figures 2 and 3. The sample containing 0.12 ml of saliva per 100 ml of solution is the only sample that represents absolute zero time, because in this experiment the lead acetate was added before the enzyme. The other "zero" time samples represent a period of 5 to 10 minutes, and they are plotted in this way on the graph.

The results of these experiments showed that increasing the time of incubation beyond two hours had little effect on the quantity of starch determined. A still shorter time of incubation was found to be adequate in other experiments which are not reported here. The samples to which lead was added after the addition of saliva, show a considerable quantity of starch, and indicate that very little time is required for the saliva to extract the starch. These data confirm the previous experiment in showing that saliva may be diluted considerably without effecting the determination of starch. In figures 2 and 3, a single line was drawn to represent the various enzyme concentrations (except at zero concentration as shown in figure 3). This

Table 2. The effect of time of incubation with saliva on the determination of starch in Abutilon leaves.

Hours of incubation	Percentage starch Ml saliva per 100 ml solution 0.12	0.60	1.2	6.0	Avg.
0	2.1	5.0	5.1	5.5	4.4
2	6.9	7.4	8.2	7.3	7.4
4	7.2	7.9	7.8	7.3	7.6
10	7.3	7.9	8.1	7.6	7.7
20	6.9	8.0	7.8	7.5	7.6
Avg. tr.	7.1	7.8	8.0	7.4	7.6

Table 3. The effect of time of incubation with saliva on the determination of starch in apple stems.

Hours of incubation	Percentage starch Ml saliva per 100 ml solution 0.00	0.12	0.60	1.2	6.0	Avg. tr.
0	2.4	7.5	8.4	8.8	9.4	8.5
2	2.2	9.3	10.0	9.8	10.8	10.0
4	2.3	9.5	9.6	10.2	10.8	10.0
10	2.3	10.0	10.2	10.4	10.8	10.4
20	2.5	9.8	11.5	11.2	11.6	11.3
Avg. tr.	2.3	9.6	10.3	10.4	11.0	10.4

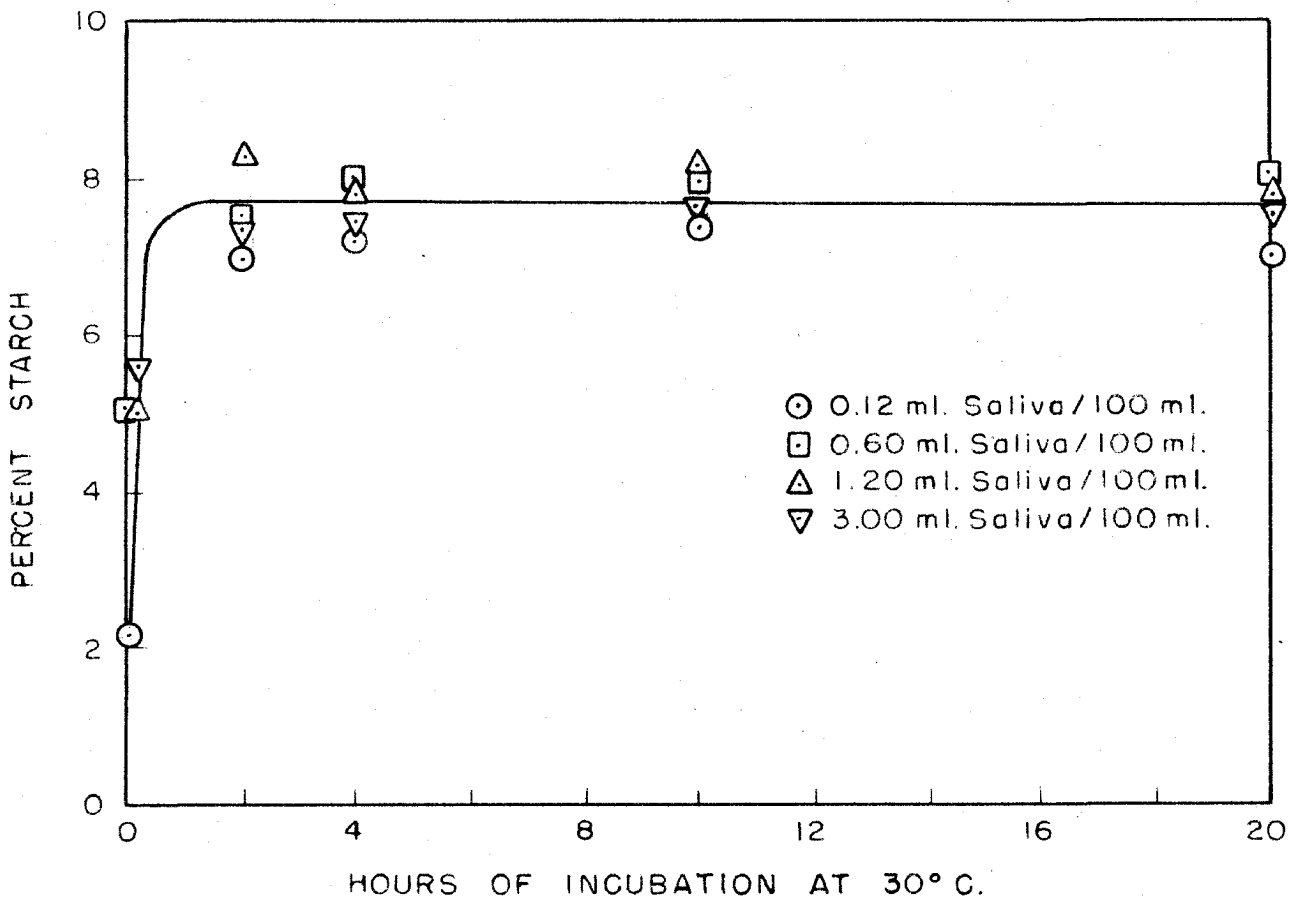

Fig. 2. The effect of the time of incubation with saliva on the determination of starch in Abutilon leaves.

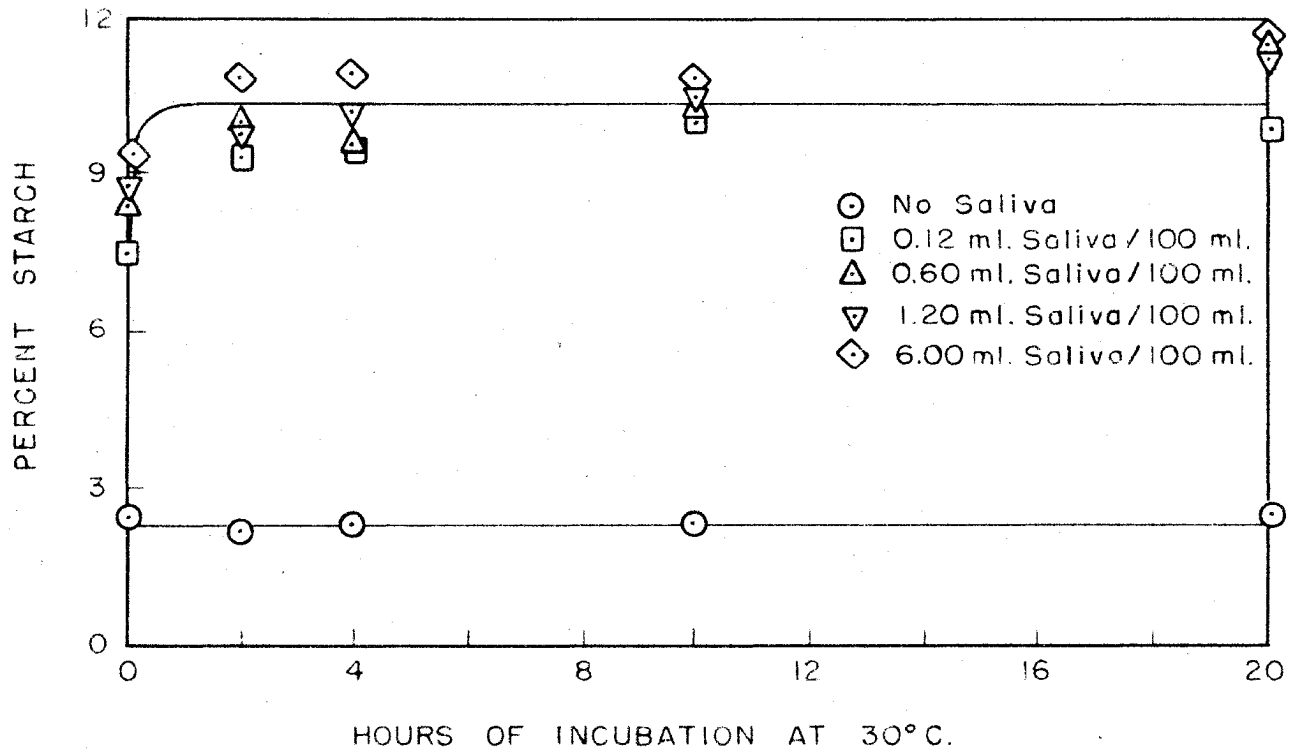

Fig. 3. The effect of the time of incubation with saliva on the determination of starch in apple stems.

was done because it was believed that the differences were not significant under the experimental conditions used. The results from the lowest concentration of the enzyme at two hours, may be somewhat low, showing that at this concentration, a longer time period of incubation is required. However, the data are insufficient to be conclusive.

Effect of taka-diastase concentration

Experiments were performed to test the effectiveness of taka-diastase in the determination of starch. These experiments were carried out in a manner similar to that used in the work on the effect of saliva concentration. Solutions containing 10 and 50 mg of taka-diastase per ml were prepared and diluted to the desired concentrations. One ml of the appropriate dilution was added to 40 ml of the gelatinized suspension of plant material, and the walls of the vessel rinsed with 9.0 ml of water to give a total volume of 50 ml. The samples were incubated for two hours, cleared and deleaded as before. The hydrolysis and sugar determination were carried out as previously described. Both apple stems and Abutilon leaves ground in a ball mill for 12 hours were used in this experiment. A blank determination was run for each concentration. No significant difference was observed for blanks at the various concentrations. Table 4 and figure 4 show the results of this

Table 4. The effect of taka-diastase on the determination of starch.

Mg taka-diastase per 100 ml of solution	Percentage starch		
	Abutilon leaves	Apple stems	Avg.
0.02	3.3	5.2	4.2
0.20	5.4	8.5	7.0
1.0	6.5	10.8	8.6
2.0	6.6	10.4	8.5
5.0	7.2	11.1	9.2
10.0	7.4	11.5	9.4
Avg.	6.1	9.6	7.8

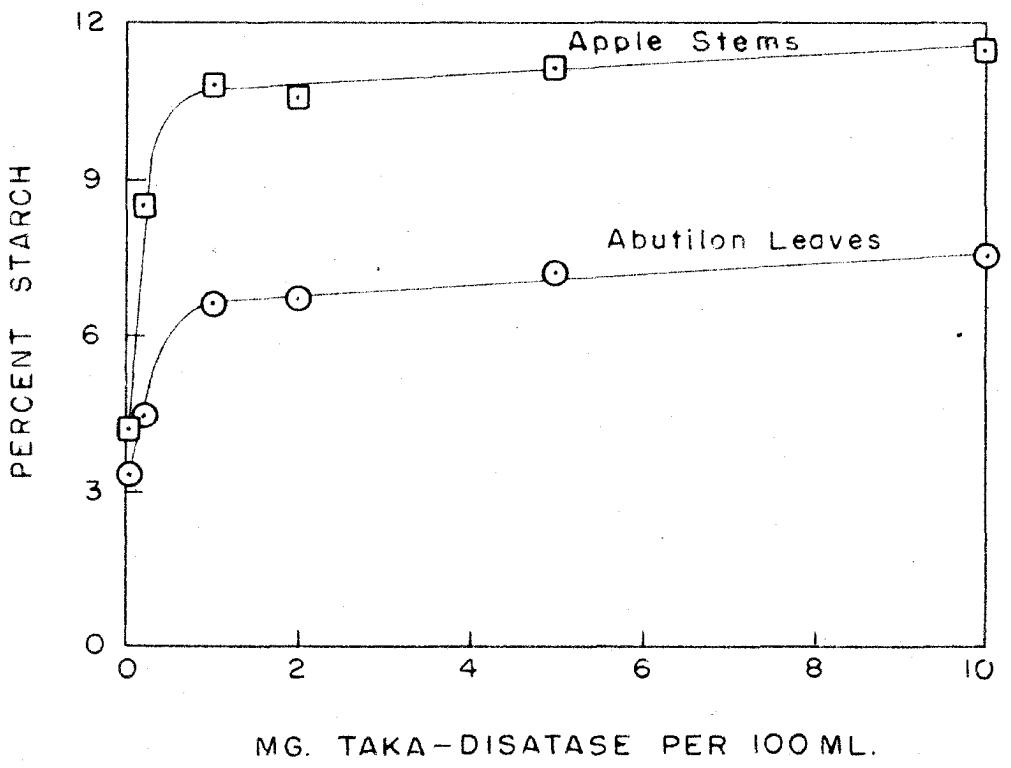

Fig. 4. The effect of taka-diastase concentration on the determination of starch.

experiment. The apparent increase in starch with increasing concentration of taka-diastase may not be significant, but this trend was noted with both apple stems and Abutilon leaves, and the experiment was performed using a more refined technique (phosphate concentration kept low) than in the experiment using saliva. It is therefore probable that this trend is significant and that it represents digestion of non starch by this mixed enzyme.

Clearing and Deleading

Many workers omit the clearing and deleading steps in determining starch. It was, therefore, thought desirable to show whether this step in the process is necessary. If clearing were not required, there would be no need for deleading. On the other hand, if clearing is necessary, it is important to know the effect of the deleading reagent on the determination.

Clearing of the plant extract

Abutilon leaves, ground in a ball mill for 12 hours or ground in the micro Wiley mill, were used in this experiment because it was noted in previous determinations that a considerable quantity of precipitate was formed upon the addition of lead acetate. If a need for clearing could be shown with one plant tissue, this step could not be eliminated

in working with any other plant tissue unless it was proven that such an ommission had no effect. The samples were weighed into 100 ml volumetric flasks, suspended and gelatinized. They were then incubated for two hours with 3 ml of 50 percent saliva. Lead acetate was added to the samples

Table 5. Effect of lead acetate as a clearing agent before acid hydrolysis in the determination of starch in Abutilon leaves.

Ml saturated lead acetate solution	Percentage starch		
	Ground in ball mill	Ground in Wiley mill	Avg.
0.0	16.4	15.2	15.8
0.5	9.5	8.6	9.0
1.0	9.3	8.6	9.0
2.0	9.0	8.2	8.6
Avg.	11.0	10.2	10.6

in the amounts of 0, 0.5, 1.0 and 2.0 ml of saturated solution. After being made up to volume, the samples were filtered into flasks containing dibasic potassium phosphate trihydrate. There was no phosphate in the flask receiving the sample to which no lead had been added. The procedure was carried through as previously described. The results are shown in table 5 and figure 5. No blank determination

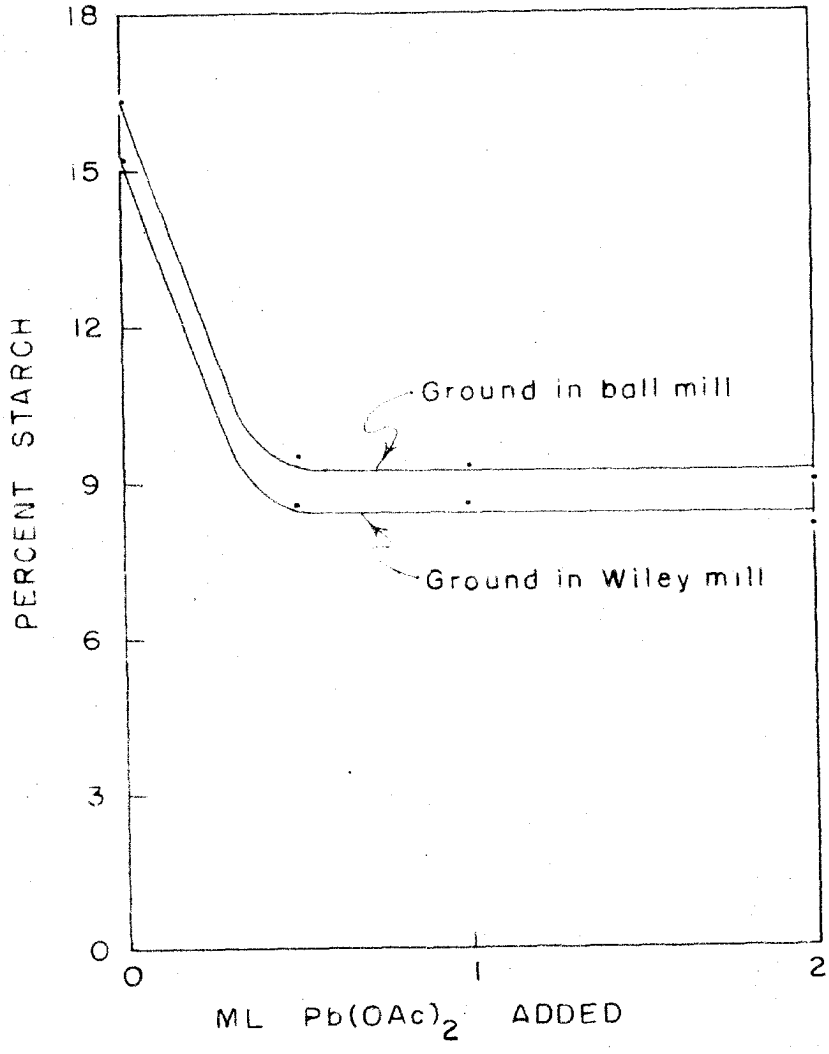

Fig. 5. The effect of lead acetate as a clearing agent in the determination of starch in Abutilon leaves.

was made in this experiment. Whereas a blank containing no lead might correct for the error, it would have to be so large that it would greatly decrease the sensitivity of the method.

Since the addition of lead acetate was necessary, the effect of the time that the samples were allowed to stand before deleading was determined. The time required to filter the cleared extract and delead is never more than one hour, and usually only about 30 minutes. An experiment using pure starch was performed. Potato starch was weighed into 250 ml volumetric flasks, suspended and gelatinized. After cooling, 7.5 ml of saliva was added and the solutions made up to volume. Following a two hour incubation under toluene, 40 ml aliquots were pipetted into 100 ml volumetric flasks and 1 ml of lead acetate was added to each sample. The various samples were allowed to stand for 0, 15, 30 and 60 minutes before filtering into flasks containing the deleading agent. The filtrate was treated in the usual manner, and the results are shown in table 6. These results show that for a 60 minute period, the lead had no adverse effect upon the determination of starch.

Deleading with dibasic potassium phosphate

An experiment was designed to show if adding an excess of the phosphate or allowing the deleaded samples to stand in

Table 6. The effect of standing in the presence of the lead acetate clearing agent on the determination of starch.

Time of standing in minutes	0	15	30	60
Percentage starch	88.9	88.7	89.8	89.8

Table 7. The effect of excess phosphate and time of standing over lead phosphate on the starch determination.

Excess phosphate	Percentage starch	
	Hours standing over lead phosphate	
	1	28
Little	8.66	8.77
Large	9.56	9.77

the presence of the lead phosphate had any effect upon the partially hydrolyzed starch present. Abutilon leaves ground in the ball mill for 12 hours were used. They were handled in the usual way except that only a small amount of solid dibasic potassium phosphate trihydrate was used with four samples, and a known large excess was used with a second four samples. After the filtration of the cleared plant extract the four samples containing a small amount of phosphate were tested for an excess of phosphate by the addition of lead acetate to a 1 ml portion. A few crystals of the phosphate were added until a slight excess was noted by this test. The determination was completed immediately on two of these samples and on two of the samples containing the large excess of phosphate. The other two of each set were saved until the next day, and the determination completed. No blank was run in this experiment. Table 7 shows that the results were not affected if the samples were allowed to stand over the lead phosphate for 28 hours, but that an excess of phosphate seemed to cause an increase in the starch content.

Since an excess of phosphate seemed to give higher starch values, another experiment was performed to check this effect. Pure starch was suspended in a 500 ml volumetric flask, gelatinized, incubated with the usual concentration of saliva for two hours, and 12 ml of saturated,

neutral lead acetate solution was added. The solution was made to volume and centrifuged. The centrifugate was somewhat opalescent, so a portion of it was filtered, but the filtrate appeared the same. Therefore, the solution was used without further treatment. Forty milliliter aliquots were pipetted into 100 ml volumetric flasks containing 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 ml of a solution of dibasic potassium

Table 8. The effect of excess phosphate on the determination of starch.

Ml $K_2HPO_4.3H_2O$ solution (1 gm per ml)	0.2	0.4	0.6	0.8	1.0	2.0
Percentage starch	89.8	90.8	94.4	98.7	100.9	103.5

phosphate. This solution contained 1 gm of the hydrate per ml and was of such a strength that 0.2 ml would just precipitate the lead ion present according to the equation:

$$3Pb^{++} + 2K_2HPO_4 \longrightarrow Pb_3(PO_4)_2 + 4K^+ + 2H^+ .$$

Qualitative tests showed that only a very slight excess of phosphate was present in the samples containing 0.2 ml of the phosphate solution. The flasks were made up to volume, and the determination carried out as usual. The results given in table 8 show that an increase in phosphate caused an increase

in the apparent percentage of starch. Since the starch used assayed 89.5 percent by direct acid hydrolysis, these data show that care must be taken not to add more than a slight excess of phosphate in the deleading process.

Acid Hydrolysis

After acid hydrolysis the acid is neutralized with sodium hydroxide, producing sodium chloride if hydrochloric acid is used and sodium sulfate if sulfuric acid is used. Weintraub and Price (27) found that various salts affected the determination of sugars with either ferricyanide or copper reagents. They found that the approximate minimal concentration causing a 2 percent error in the determination of 2 mg glucose, was 0.25 N for sodium chloride and 0.22 N for sodium sulfate. Hydrochloric acid was used in the acid hydrolysis in the starch determination being studied. In view of these findings of Weintraub and Price, it is important to keep the hydrochloric acid concentration low enough, so that when the neutralized solution is made to volume before the glucose determination, the chloride ion concentration does not exceed 0.25 N.

In the technique used in this work, 40 ml of solution was hydrolyzed, neutralized and made up to 50 ml. Assuming that concentrated hydrcchloric acid is 12.0 N, 1 ml of the concentrated acid will give a final concentration of 0.24 N.

If a larger volume of the concentrated acid is used, the final dilution must be larger. A dilution greater than 40 ml of solution to a final volume of 50 ml, is undesirable because it decreases the sensitivity of the method. It was therefore thought important to determine the concentration of hydrochloric acid that would bring about complete hydrolysis in a reasonable length of time. Potato starch was weighed into a liter volumetric flask, suspended in water and gelatinized. This suspension was digested with saliva, cleared and deleaded. After making the solution to volume, 40 ml portions were pipetted into 50 ml volumetric flasks and 0.00, 0.25, 0.50 and 1.00 ml of concentrated hydrochloric acid was added to various samples. Different samples were hydrolyzed at 15 pounds pressure for 30 and 60 minutes, and at five pounds for 50 and 100 minutes, cooled in a water bath, neutralized and made to volume. Glucose was determined on these samples. The results are shown in table 9. These data show that 0.5 ml of concentrated hydrochloric acid, or 1 + 80, is sufficient to bring about maximum hydrolysis at 15 pounds in one hour.

Gelatinization

In the method being studied here, the starch is gelatinized before being digested with an enzyme. This process not only gelatinizes the starch, but also helps to free it from the cells, thus making it available to the enzyme.

Table 9. The effect of hydrochloric acid concentration on the hydrolysis of starch after digestion with saliva.

Ml HCl added to 40 ml extract	Pounds pressure	Minutes in autoclave	Percentage starch
0.00	5	50	58.9
0.00	5	100	57.2
0.00	15	30	58.6
0.00	15	60	59.1
0.25	5	50	71.9
0.25	5	100	77.2
0.25	15	30	80.8
0.25	15	60	87.1
0.50	5	50	81.2
0.50	5	100	85.3
0.50	15	30	88.0
0.50	15	60	90.1
1.00	5	50	87.5
1.00	5	100	89.0
1.00	15	30	88.4
1.00	15	60	90.6

The effect of such treatment was studied. Apple stems ground in a Wiley mill with an 80 mesh screen and Abutilon leaves ground in the same mill with a 40 mesh screen were used. Samples were weighed, suspended in 40 ml of water and gelatinized under various conditions, as shown in table 10. The analysis was then carried through in the usual manner. In addition, samples of pure starch were heated in a boiling water bath for 30 minutes and autoclaved at 15 pounds pressure for 60 minutes. This work shows that starch is not affected by the gelatinization conditions, and that for the material studied, a 30 minute gelatinization was sufficient. Fifteen pounds pressure for 30 minutes was decided upon, since this was not harmful to the starch and allows some margin of safety for more refractory tissue. In addition, this treatment will sterilize the samples and eliminate one source of microorganisms that might affect the method.

Effect of Grinding

As suggested previously and confirmed by some early experiments, the grinding of the plant material is probably an important factor in the extraction of starch. Abutilon leaves and stems were ground as follows:

Ball mill for four hours

Ball mill for 24 hours

Micro Wiley mill with a 40 mesh screen

Table 10. The effect of various conditions of gelatinization on the determination of starch.

Plant material	Conditions of gelatinization	Percentage starch
Apple stems	No gelatinization	7.0
Apple stems	10 lbs. for 30 min.	9.6
Apple stems	15 lbs. for 30 min.	10.1
Apple stems	15 lbs. for 60 min.	9.8
Apple stems	15 lbs. for 90 min.	10.1
Apple stems	20 lbs. for 30 min.	9.5
Abutilon leaves	15 lbs. for 30 min.	6.9
Abutilon leaves	15 lbs. for 60 min.	7.0
Starch	Boiling water bath for 30 min.	92.0
Starch	15 lbs. for 60 min.	93.1

C and M laboratory mill, number 8, with approximately 60 mesh screen

No treatment (samples of Abutilon stems had been previously ground in a Wiley mill using a coarse screen)

The samples were screened through U. S. standard sieves. The distribution of particles is shown in table 11. The various samples of Abutilon stems were weighed into centrifuge tubes and suspended in 30 ml of 10 percent alcohol. Each sample was centrifuged and the clear centrifugate decanted off. This process was repeated three times to extract the dextrins. The insoluble material was washed into a 250 ml flask with the smallest possible volume of water, steamed in the autoclave for 30 minutes, cooled, and 3 ml of saliva was added. After incubation of the starch fraction at 37°C. for 20 hours, it was treated in the same way as the dextrin fraction. Neutral lead acetate (1 ml) was added to each sample and the material quantitatively filtered into a 250 ml volumetric flask containing a solution of K_2HPO_4, and diluted to volume. The lead phosphate was allowed to settle. A 40 ml aliquot was pipetted into a 50 ml volumetric flask, and hydrolyzed by addition of 2 ml of concentrated hydrochloric acid and autoclaving at 15 pounds for 60 minutes. The flask was cooled, neutralized and made to volume. Glucose was determined on this hydrolysate, and the starch and dextrin calculated. A conversion factor of 0.9 was used for dextrins as well as for starch.

Table 11. Particle distribution of Abutilon tissue after grinding.

Sieve size	Percentage				
	Ball mill 4 hours	Ball mill 24 hours	Wiley mill	C and M mill	No treatment
			Stem material		
>20	0.2			0.1	8.4
20-40	16.4		0.1	6.0	51.1
40-80	45.4	0.3	60.4	64.5	28.8
80-120	18.2	0.6	20.6	19.4	5.6
120-200	9.4	3.3	8.6	6.4	3.3
<200	10.3	96.0	9.9	3.1	2.7
Total	99.9	100.2	99.6	99.5	99.9
			Leaf material		
>25				0.1	74.0
25-50	0.1		17.6	3.1	19.2
50-100	6.6	0.3	59.5	46.7	3.4
100-200	11.6	1.7	11.0	28.8	0.8
<200	71.4	98.0	11.8	21.3	2.8
Total	99.7	100.0	99.9	100.0	100.2

The Abutilon leaf material was analyzed for starch and dextrin in the same way, except that the starch fraction was gelatinized and incubated in the centrifuge tubes. After clearing with lead acetate, the solution was filtered into a 100 ml volumetric flask, deleaded and made to volume. The results of these experiments are shown in table 12.

These experiments were done before the other work presented in this paper, with slightly different methods, and are comparable only within themselves. It is significant, however, that in the samples ground for 24 hours in the ball mill, the dextrin content was high and the starch content low. The total starch plus dextrin agreed within the experimental error, except for the Abutilon stems ground in the C and M mill. The low value obtained here was probably due to selective loss of the finner material in grinding. In view of these results, the effect of grinding in a ball mill on pure starch was studied. Pure starch was ground for 0, 4, 8, 12 and 20 hours in the ball mill. Samples were weighed into centrifuge tubes, suspended in 20 ml of 10 percent alcohol, and centrifuged. This extraction was repeated three times, and the combined centrifugates poured into a 100 ml volumetric flask. It was noted, at this point, that the samples subjected to ball milling were somewhat gelatinous, the degree depending upon the length of grinding. The insoluble starch fraction was gelatinized and

Table 12. Effect of grinding on the starch and dextrin analyses of Abutilon tissue.

Method of grinding	Percentage dextrin	Percentage starch	Total
		Stem tissue	
Ball mill, 4 hr.	0.66	4.1	4.8
Ball mill, 24 hr.	1.2	3.4	4.6
Wiley mill	0.50	4.0	4.5
C and M mill	0.52	3.0	3.5
No treatment	0.45	5.0	5.5
		Leaf tissue	
Ball mill, 4 hr.	.54	2.7	3.2
Ball mill, 24 hr.	1.68	2.0	3.7
Wiley mill	.37	3.0	3.4
C and M mill	.20	3.4	3.6
No treatment	.13	3.2	3.3

quantitatively transferred to 100 ml volumetric flasks. Both fractions were hydrolyzed with 1 ml of concentrated HCl by heating for 60 minutes at 15 pounds pressure in an autoclave. The samples were then cooled, neutralized and made to volume. Glucose was determined and the starch and dextrin calculated. The results in table 13 and figure 6

Table 13. The effect of grinding in a ball mill on potato starch.

Hours in ball mill	Percentage dextrin	Percentage starch	Total
0	0.9	90.0	90.9
4	5.5	87.2	92.7
8	6.5	83.2	89.7
12	8.4	80.3	88.7
20	65.2	18.1	83.3

show rather conclusively the effect of excessive grinding on the solubility and presumably the molecular size of starch.

Three methods of grinding were tested with apple stems. The dried material was ground in a ball mill for 12 hours, in a Wiley mill with an 80 mesh screen and in a Wiley mill

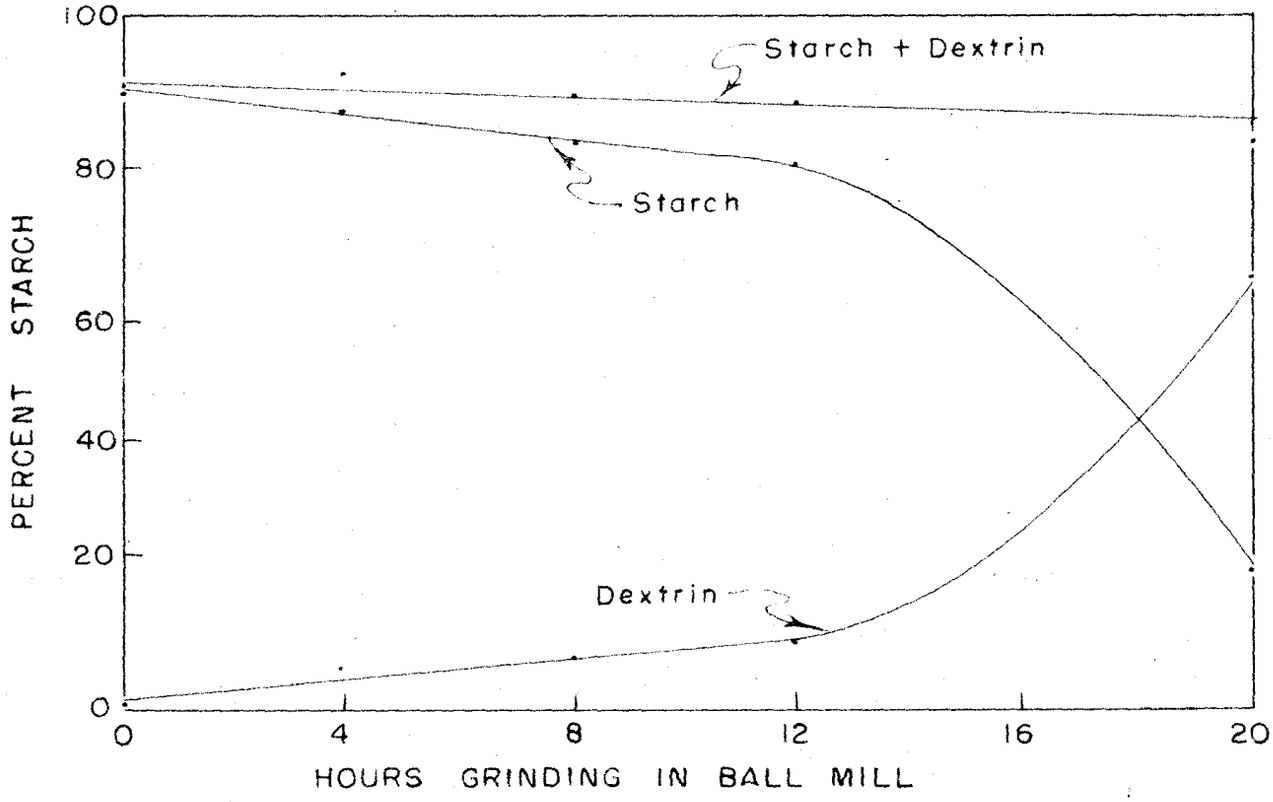

Fig. 6. The effect of grinding on the starch-dextrin distribution in potato starch.

with a 40 mesh screen. The samples were weighed into centrifuge tubes, suspended in 30 ml of 10 percent alcohol and centrifuged. The clear centrifugate was decanted into 100 ml volumetric flasks. This process was repeated three times using 20 ml of 10 percent alcohol. The combined centrifugates were cleared, made to volume, filtered and deleaded. The dex-

Table 14. Effect of various methods of grinding on the determination of starch and dextrin in apple stems.

Grinding	Percentage dextrin	Percentage starch	Total
Ball mill, 12 hours	2.02	7.29	9.31
Wiley mill with 80 mesh screen	0.36	8.44	8.80
Wiley mill with 40 mesh screen	0.07	8.00	8.07

trin present in a 40 ml aliquot was hydrolyzed with acid and the glucose produced was determined. The starch present in the 10 percent alcohol insoluble fraction was determined as previously described. The results are reported in table 14. These results again show that increased grinding caused an increase in the dextrin fraction

and a decrease in starch. In this case, however, the more vigorous methods of grinding seemed to show an increase in the total starch plus dextrin content, which may represent a better removal of starch or a partial removal of hemicellulose material.

A Method of Starch Determination

A method of starch determination derived from the results of the experiments presented in this paper will be given in detail here. A sample of dry material that has been extracted with 80 percent alcohol to remove sugars is weighed to the nearest milligram. The sample size should be large enough to contain 20 to 50 mg of starch. If it is desirable to separate dextrins from starch, the sample is weighed into a centrifuge tube and extracted four times by suspending the plant material in 10 percent alcohol and centrifuging. The combined centrifugates (dextrin fraction in 10 percent alcohol) are poured into a 100 ml volumetric flask. The residue from this extraction is transferred quantitatively to a 100 ml volumetric flask with about 40 ml of water. If the starch and dextrin fractions are to be determined together, the sample is weighed directly into the volumetric flask and suspended in 40 ml of water[1]. If the material is difficult to wet, the

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[1]This is frequently permissible since in many plant tissues dextrins do not amount to more than 1 percent.

vacuum infiltration technique described previously may be used. The suspension is gelatinized in the autoclave at 15 pounds pressure for 30 minutes and allowed to cool or cooled in a water bath. To the cooled sample, 3 ml of filtered 50 percent saliva is added, and it is incubated at 30°C. for 2 to 3 hours. From this point, (i. e. after incubation), the starch and dextrin are treated in the same way. One ml of a solution of saturated neutral lead acetate is added and the sample diluted to the 100 ml mark with water, shaken and filtered through a fluted, starch free filter paper (Whatman number 30 was used in this work) into a 125 ml flask containing about 0.2 gm of $K_2HPO_4.3H_2O$. After the sample has filtered, it is shaken and tested for the presence of an excess of phosphate by adding a drop of lead to a few milliliters of the deleaded solution. A precipitate forms if an excess of phosphate is present. If the test is negative, a few crystals of phosphate are added so that only a slight excess of phosphate is present. The lead phosphate present in the sample is centrifuged down or allowed to settle out over night. This is a good point to stop if the analysis can not be finished in one day since standing for at least 28 hours has no adverse effect. A 40 ml aliquot of the clear solution is pipetted into a 50 ml volumetric flask and 0.5 ml of concentrated HCl added. The solution is mixed and hydrolyzed by heating

in an autoclave at 15 pounds for 60 minutes. After the solution has cooled, it is neutralized with 15 percent NaOH using methyl red as an indicator. The exact methyl red end point (a faint pink) is obtained by a final adjustment of the pH with dilute HCl and NaOH. After diluting the solution to 50 ml with water, glucose is determined. A blank determination is run, in which boiled saliva is substituted for fresh saliva and the acid hydrolysis is omitted. The percentage starch is calculated by the formula:

$$\text{Percent starch} = \frac{0.9G}{W} \times 100$$

where G is the milligrams of glucose per sample and W is the weight of the sample in milligrams.

Analysis of various plant materials

Several plant tissues were ground in a Wiley mill with an 80 mesh screen and assayed for starch and dextrin by the above method. In addition, determinations were made on the starch in which the usual concentration of HCl was used, but boiled saliva was substituted for fresh saliva. The results of these experiments are shown in table 15.

Table 15. Estimation of starch and dextrin in various plant tissues.

Plant material	Percentage dextrin		Percentage starch		
	Assay	Blank	Assay	Without saliva	Blank
Onion bulb	2.07	0.42	1.16	1.22	0.36
Turnip	1.01	0.24	4.61	1.50	0.69
Portulaca	1.71	0.26	9.85		0.79
Tomato stems	0.20	0.11	6.40	2.06	1.31
Alfalfa roots	1.08	0.28	20.00	3.55	0.47
Abutilon roots	0.18	0.05	1.62	0.84	0.37
Abutilon stems	0.36	0.19	8.42	2.64	0.54
Abutilon leaves	0.31	0.14	6.51	2.05	0.75

DISCUSSION

A large number of carbohydrates are found in plants, and most of these may interfere with the determination of starch. Among these compounds are monosaccharides, disaccharides, pectins, gums and hemicelluloses. Cellulose and lignin are very prevalent but not apt to interfere. In addition, any substance which will reduce ferricyanide will give high results. Many such substances, as well as low molecular weight carbohydrates, may be removed by a preliminary extraction with 80 percent alcohol.

Clearing with neutral lead acetate will remove any acidic substances such as gums and pectins. The size of the error which may be obtained if clearing is omitted is shown in table 5 and figure 5. Nearly half of the reducing materials extracted from Abutilon leaves after gelatinizing and treating with saliva was precipitated with neutral lead acetate, indicating the presence of pectins and gums. These substances may be of physiological importance, but they should not be reported as starch.

When reducing substances are used to estimate starch, it is necessary to remove the lead rather completely. Dibasic potassium phosphate is useful for deleading, but our results (table 8) show that any considerable excess

of phosphate must be avoided. Weintraub and Price (27) show that the effect of excess phosphate is on the glucose determination. They show also that the effect is as great with copper methods of sugar analysis as with ferricyanide.

The effect of preliminary grinding of the tissues on the ease and completeness of starch extraction, was studied in many comparisons. In general, grinding to 20-40 mesh in a Wiley mill gave reasonably good recovery, although significant increases in the starch plus dextrin were shown by leaf and stem tissues ground in a ball mill (tables 5, 14). The exact cause of these increases is not clear, however. First, they are small, and second, they could represent the solubilization of hemicelluloses or similar materials.

The data of table 13 and figure 6 show conclusively that potato starch is in some way changed during grinding in a ball mill. This change could be either in the physical properties of the starch or an actual rupture of the bonds. The latter explanation seems probable to the author because the values of blank determinations were significantly increased in the 12 and 20 hour grinding, which indicates the formation of reducing substances. The decrease in the starch plus dextrin with increased time of grinding was in part due to the increase in the blank, but probably chiefly due to a greater ash content from the mill and its flint charge. The change in starch on grinding

in the ball mill occurred also in the plant material as shown in tables 12 and 14. In addition, the data in table 14 show that for apple stems, higher starch plus dextrin values were obtained with increased grinding. It might be expected that increased grinding would aid in the extraction of starch, but it is equally true that interfering substances would also become more readily extracted. If starch is changed by the action of the ball mill, it is reasonable to assume that non-interfering substances also might be changed in such a way as to become interfering. Thus there are two conflicting factors to consider in the grinding of plant material. Insufficient grinding will lead to low results by making it impossible to extract the starch completely. Too much grinding will lead to low results by bringing about changes in the starch or possibly to high results due to the introduction of interfering substances. As stated above, a moderately fine grinding would seem to be adequate for most tissues.

The need for gelatinization prior to treatment with diastase in the determination of starch is shown by the data in table 10. The percentage starch obtained in apple stems was increased about 40 percent by gelatinization. Neither the minimum amount of time or temperature was determined. A various times heating in a water bath was tried, but the results were inconsistant. The author believes

that some pressure is desirable, and it was shown that heating starch for 60 minutes at 15 pounds pressure was not excessive. Such a gelatinization treatment might be expected to effect some hydrolysis of starch. The data in tables 15 and 3 substantiate this. The data in table 3 were obtained with a 60 minute gelatinization and those in table 15 with a 30 minute gelatinization. In both cases some "starch" was extracted without diastase.

The necessity of enzymatic digestion is shown in tables 1, 2, 3 and 15 and figures 1, 2 and 3. Since the treatment is followed by acid hydrolysis, it is not essential that the enzyme hydrolysis proceed to completion. Tables 1, 2 and 3 and figures 1, 2 and 3 show that only a small quantity of enzyme and a short period of incubation is necessary for salivary diastase to break down starch sufficiently to pass through a filter. Long periods of incubation increase the possibility of microbiological activity. On one run with samples incubated under toluene, the assay value after 24 hours was only about half that obtained after two hours. Toluene may at times fail as a preservative, and a long incubation increases this danger. Saliva is preferable to taka-diastase in the determination of starch because the latter is a complex mixture of enzymes and will hydrolyze substances other than starch. In addition, commercial taka-diastase is diluted with lactose and must

be dialyzed before use.

The recovery of starch was dependent upon the concentration of hydrochloric acid, temperature of the hydrolysis and the length of time of the hydrolysis as is shown in table 9. The concentration is probably the most important of these since this is the factor which determines the sodium chloride concentration resulting on neutralization. Increased sodium chloride will cause high results in the glucose determination. It is, therefore, better to use a higher temperature for a longer time and keep the HCl concentration at a minimum. This condition is reached with 0.5 ml of HCl added to 40 ml of extract and heated in the autoclave for 60 minutes with 15 pounds pressure.

The data of table 15 show that there may still be difficulties in the estimation of starch with the best methods available. Onion and turnip tissues gave negative tests for starch with iodine, but showed "starch" with our modification of the Loomis and Shull method. Both tissues showed some charring after drying, and hemicelluloses or non-acidic gummy materials may have been so changed by this heating as to interfere later. In the starch determination on onion, the extraction by gelatinization only was as high as that with salivary diastase, suggesting that non-starch was involved.

Small quantities of amylopectin might be overlooked in

a microscopic test for starch, but the turnip tissue assayed 4.6 percent of apparent starch. Further work will be necessary to clarify the reactions involved.

SUMMARY

The various factors affecting the estimation of starch by determining the glucose formed by acid hydrolysis following enzymatic hydrolysis have been studied. The effect of grinding of the plant material was shown to be important. Insufficient grinding leads to low values in starch, and grinding in the ball mill caused a breakdown of starch, and probably of non-starch interfering substances, to more soluble material.

Gelatinization is necessary to extract starch from plant tissue. Probably vigorous conditions are not needed, but autoclaving for one hour at 15 pounds had no detrimental effect on starch recovery.

Salivary diastase is preferred to taka-diastase in the enzymatic digestion. It is better to use a short incubation time to decrease the danger of bacterial activity.

Clearing of the enzymatic extracts is essential to remove certain interfering substances. Even when this is done, however, not all the interfering substances are removed and results are sometimes high. After clearing, it is necessary to delead. It is important not to use too large an excess of K_2HPO_4 for this purpose.

In the acid hydrolysis the HCl concentration must be kept low enough so that the NaCl formed on neutralization

will not give a high sugar titration. The condition necessary for complete hydrolysis is 1 + 80 HCl heated in an autoclave at 15 pounds pressure for 30-60 minutes.

Certain optimum conditions were determined for the estimation of starch:

Grinding to pass through a 40 to 80 mesh screen

Gelatinization in an autoclave for 30 minutes at 15 pounds pressure

Incubation of the sample with $\frac{4}{-}$ 3ml of 50 percent saliva for two hours

Clearing with lead acetate, filtering and deleading with a slight excess K_2HPO_4

Hydrolysis of the extract with 1 + 80 HCl at 15 pounds for 30-60 minutes

Determination of the reducing sugars formed.

LITERATURE CITED

1. Baldwin, R. R., Bear, R. S., and Rundle, R. E. The relation of starch-iodine absorption spectra to the structure of starch and and starch components. J. Am. Chem. Soc. 66:111-115. 1944.

2. Bates, F. L., French, D., and Rundle, R. E. Amylose and amylopectin content of starches determined by their iodine complex formation. J. Am. Chem. Soc. 65:142-148. 1943.

3. Clendenning, K. A. Polarimetric determination of starch in cereal products. IV. Critical studies of methods for the determination of starch in whole wheat, granular, and patent flours. Can. J. Research. 23B:239-259. 1945.

4. Collins, J. D. Quantitative hydrolysis of starch by buffered taka-diastase. Science 66:430-431. 1927.

5. Davis, W. A. and Daish, A. J. Methods of estimating carbohydrates. II. The estimation of starch in plant material. The use of taka-diastase. J. Agr. Sci. 6:151-168. 1914.

6. Denny, F. E. Improvements in the methods of determining starch in plant tissues. Contrib. Boyce Thompson Inst. 6:129-146. 1934.

7. Denny, F. E. Starch determination methods involving solubility in acids. Contrib. Boyce Thompson Inst. 6:381-393. 1934.

8. Fellenberg, T. von. Eine derekte, allgemein anwendbare Stärkebestimmungsmethode. Mitt. Gebiete Lebensm. u. Hyg. 7:369-383. 1916.

9. Fraps, G. S. Estimation of starch in feeding stuffs. J. Assoc. Offic. Agr. Chemists. 15:304-307. 1932.

10. Griffiths, D. G. and Potter, N. A. The determination of starch in apple tissue. Biochem. J. 39:423-426. 1945.

11. Hassid, W. Z. Determination of sugars in plants by oxidation with ferricyanide and ceric sulfate titration. Ind. Eng. Chem., Anal. Ed. 9:228-229. 1937.

12. Hassid, W. Z., McCready, R. M., and Rosenfels, R. S. Determination of starch in plants. Ind. Eng. Chem., Anal. Ed. 12:142-144. 1940.

13. Hermbstaedt. Versuche und Beobachtungen über die chemische Zergliederung vegetabilischorganischer Erzeugnisse überhaupt und der Getreidearten insbesondere; mit Rücksicht des Einflusses der Düngungsmittel auf die Bestandtheile der Letztern. J. Tech. u. Öknomische Chemie von Otto Linne' Erdmann. 12:1-53. 1831.

14. Horton, E. O. On the use of taka-diastase in estimating starch. J. Agr. Sci. 11:240-257. 1921.

15. Lind, E. F. Clearing and deleading of plant sugar extract. Unpublished M. S. Thesis. Ames, Iowa, Iowa State College Library. 1947.

16. Lind, E. F. Clearing and deleading of plant extracts for reducing sugar determinations. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library. 1949.

17. Loomis, W. E. and Shull, C. A. Methods in plant physiology. New York, McGraw-Hill Book Company, Inc. 1937.

18. Nielsen, J. P. Rapid determination of starch. An index to maturity in starchy vegetables. Ind. Eng. Chem., Anal. Ed. 15:176-179. 1943.

19. Nielsen, J. P., and Gleason, P. C. Rapid determination of starch. Factors for starches and comparison with acids and enzymatic hydrolysis methods. Ind. Eng. Chem., Anal. Ed. 17:131-134. 1945.

20. Noyes, W. A., Crawford, G., Jumper, C. H., Flory, E. L., and Arnold, R. B. The hydrolysis of maltose and of dextrin by dilute acids and the determination of starch. J. Am. Chem. Soc. 26:266-280. 1904.

21. Pucher, G. W., Leavenworth, C. S., and Vickery, H. B. Determination of starch in plant tissues. Anal. Chem. 20:850-853. 1948.

22. Pucher, G. W., and Vickery, H. B. Determination of starch in plants with special reference to woody plants. J. Assoc. Offic. Agr. Chemists. 18: 621-636. 1935.

23. Rask, O. S. A rapid method for the determination of starch. J. Assoc. Offic. Agr. Chemists. 10:108-120. 1927.

24. Schoch, T. J. Fraction of starch. Advances in Carbohydrate Chem. 1:247-277. 1945.

25. Sullivan, J. T. The estimation of starch. Ind. Eng. Chem., Anal. Ed. 7:311-314. 1935.

26. Sullivan, J. T. The estimation of starch in plants with special reference to woody plants. J. Assoc. Offic. Agr. Chemists. 18:621-636. 1935.

27. Weintraub, R. L., and Price, L. Influence of various substances on sugar determination by copper and ferricyanide reagents. Smithsonian Inst. Pubs. Misc. Collections. 104(No.10):1-17. 1945.

28. Widdowson, E. M. A method for the determination of small quantities of mixed reducing sugars and its application to the estimation of the products of hydrolysis of starch by taka-diastase. Biochem. J. 25:863-879. 1931.

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